Early stages of development in the endangered limpet *Patella ferruginea* Gmelin, 1791 (Gastropoda: Patellidae)

Free Espinosa
Georgina A. Rivera-Ingraham
José C. García-Gómez
Laboratorio de Biología Marina
Departamento de Fisiología y Zoología
Universidad de Sevilla
Avda. Reina Mereedes 6
41012 Sevilla, SPAIN
free@us.es

ABSTRACT

The larval biology of *Patella ferruginea* is studied for the first time. Development in the species first showed two complete and equal cleavages whereas the third cleavage was unequal, resulting in an embryo with 4 micromeres and 4 macromeres. Early trochophores were detected 19 hours post-fertilization and pretorsional veligers appeared 27 hours post-fertilization. Early stages of development are very similar to those shown by other related limpet speeies, with higher developmental times than those recorded for *Patella caerulea* and similar to those obtained in *Patella vulgata*. However, *in vitro* fertilization and the obtainment of spats in a massive amount could be the solution for replenishing threatened or extinct populations of this extremely endangered Mediterranean speeies. The results of the present study represent a first approach in order to produce great amounts of spats in laboratory conditions for further reintroduction projects aiming for conservation of the species.

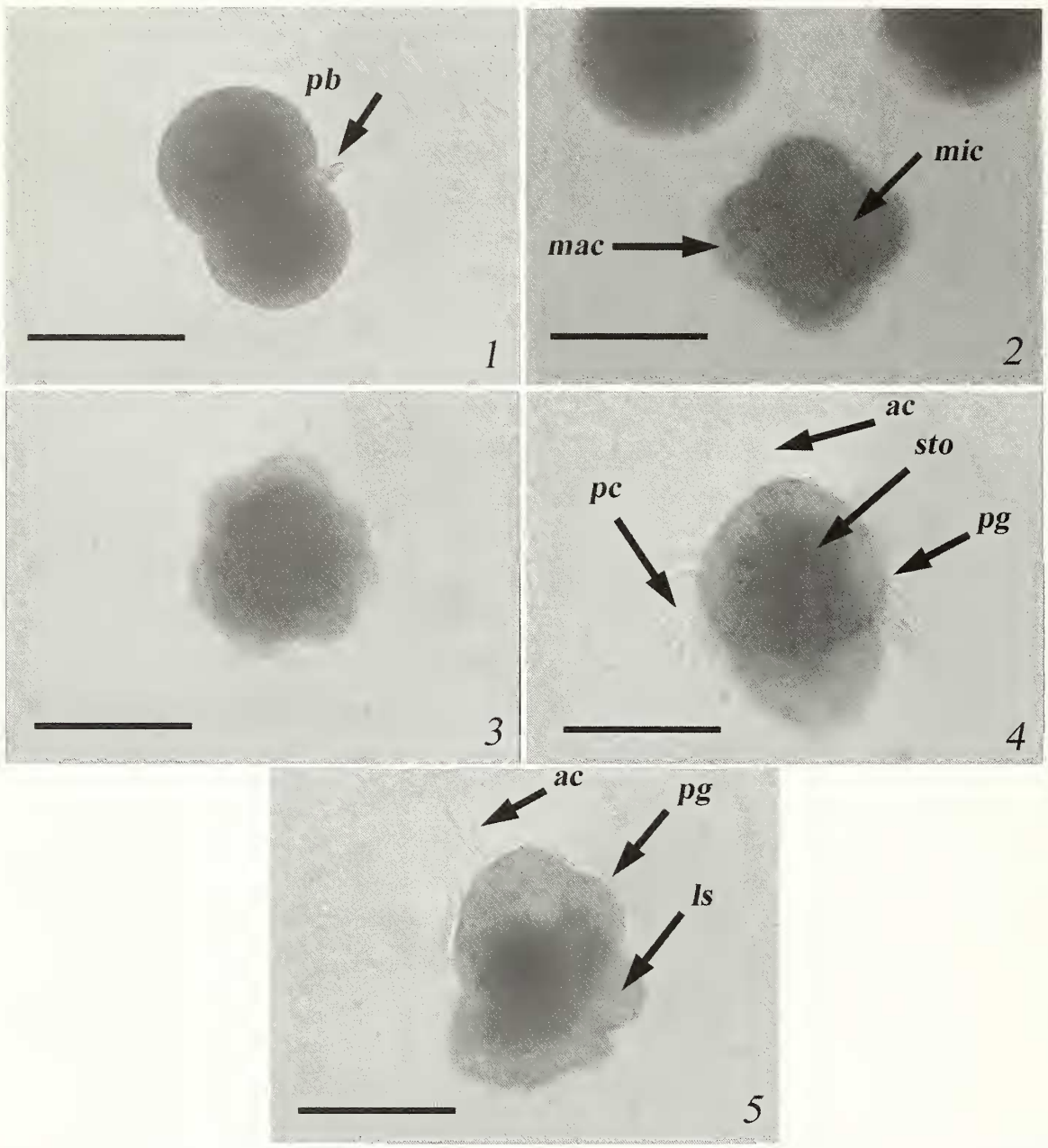
Additional keywords: Conservation, endangered speeies

INTRODUCTION

The mollusk *Patella ferruginea* Gmelin, 1791, endemie to the Mediterranean, is the most endangered marine species listed on the European Council Directive 92/43/EEC and it is presently under serious risk of extinction (Laborel-Deguen and Laborel, 1991; Ramos, 1998; Espinosa et al., 2006). Nevertheless, its biology and ecology are poorly known (Guerra-García et al., 2004) and studies mainly foeused on the reproductive biology and larval development of the species are urgently required, as pointed out by Templado (2001) and Guallart et al., (2006), in order to implement adequate management and eonservation strategies. The larval biology has not been studied in detail before, although some preliminary fertilization assays were made on the field by Guallart et al., (2006). Taking into account that in the last decades captive breeding has been suggested an important supportive intervention to avoid the loss of many speeies (IUCN, 1987), *in vitro* fertilization of *Patella ferruginea* and the obtainment of spats in a massive amount could be the solution for replenishing the threatened or extinct populations of the species around the Mediterranean. The development of appropriate culture techniques is considered to be crucial for the future conservation of the species.

MATERIALS AND METHODS

Adults of *Patella ferruginea* were collected from Ceuta, North Africa (35°53′20″ N, 5°18′30″ W), during October and November 2006, overlapping with the annual reproduction period of the species (Frenkiel, 1975) and independently of the lunar cycle, but always during low tide. Animals were kept in an aquarium, at a constant temperature of 18°C. Additionally, and in order to determine the maturation stage of the gonads, biopsies were taken. The gonads of ripe animals were dissected for artificial insemination following Van den Biggelaar (1977) and Wanninger et al. (1999) protocols; all further fertilization, culture, and breeding procedures were earried out in Seachem® artificial seawater filtered through a 0.45 µm mesh (AFSW) at 18°C. 1000 ml beakers were used as culture vessels, each filled with 500 ml of AFSW, stirred through moderate aeration. Eggs from four females were treated with alkaline sea water (pH = 8.9, by addition of drops of NH_4OH) for 1–7 minutes before fertilization to induee the egg-ripening proeess, followed by stirring, allowed to settle for 3 minutes, washing, and decanting 4 or 5 times (see also Dodd, 1957). Sperm of four males was diluted in AFSW until the suspension beeame fully clear; the agility of sperm cells was confirmed under the microscope before insemination. For fertilization, 10–20 drops of sperm suspension were used per liter of egg-eontaining AFSW. The percentage of fertilized eggs at each treatment was assessed using a compound microscope by the presenee of normal or abnormal cleavage in 100 undamaged eggs that were



Figures 1–5. Development stages in *Patella ferruginea*, reared at 18°C. Light micrographs. **1.** First cleavage (2 h) displaying polar body (**pb**). **2.** Eight-cell stage (4 h). Micromeres (**mic**); macromeres (**mac**). **3.** >12 cells: morula (5 h). **4.** Completely formed trochophore (19 h) with apical cilia (**ac**), prototrochal cilia (**pc**), prototrochal girdle (**pg**) and stomodeum (**sto**). **5.** Early pretorsional veliger (27 h) with apical cilia (**ac**), prototrochal girdle (**pg**) and larval shell (**ls**). Scale bars 100 µm.

randomly sampled from each vessel at least 4 h after insemination (see Baker and Tyler, 2001).

Larval cultures were kept in AFSW with 50 mg streptomycin and 60 mg penicillin per litre to minimize microbial or fungal infection. The water was changed periodically by pouring the vessels' content on to a sieve of 100 µm being held partly under water to prevent damage to the larvae. Larval development was monitored with a compound microscope, and major stages were recorded with photomicrographs.

RESULTS

Eggs of *Patella ferruginea* present an average diameter of 149.78 µm whereas sperm shows an average length of 3.78 µm (Espinosa et al., 2006). The first evidence of fertilization was the extrusion of the first polar body in the first two hours after insemination, and the completeness of the first two cleavages, equal and always initiated near the polar body (Figure 1). However, the fertilization rate resulted very low (3±1%). The third cleavage was

unequal and equatorial, such that the resulting 8-celled embryos were composed of 4 macromeres and 4 micromeres (Figure 2). Trocophores presented the natural morphology associated with the taxon and swam actively during 19 hours after fertilization, with telotroch (anal tuft), apical and prototrochal cilia (Figure 4) well developed and similar to those described for other patellid limpet species. In early trochophores, steady swimming was often alternated with abrupt bursts of speed. This sprint behaviour was constantly observed. Trochophores swam horizontally and vertically in culture vessels. The stomodeum was visible immediately beneath the prototrochal girdle (Figure 4). In pretorsional veliger the larval shell started to be observed (Figure 5) 27 hours after fertilization, whereas other structures remained, as the apical cilia and prototrochal girdle. This pretorsional veliger could be observed until 48 hours after fertilization, when the remained larvae died.

DISCUSSION

The early stages of larval development in *Patella ferruginea* obtained in the present study, appear to be very similar to other related limpet species, with higher developmental times than *Patella caerulea* (see Wanninger et al., 1999) and similar to those obtained in *Patella vulgata* (see Dodd, 1957; Wanninger et al., 1999). Nevertheless, it is known that water temperature influences the timing of developmental events (Kay and Emlet, 2002). In this sense, the proportional timing for *P. ferruginea* could be higher than for *P. vulgata*, although further experiments would be necessary. It is important to note that the fertilization rates obtained were very low and that the larval survival did not exceed 48 h, despite the use of similar methods that have reported good results in other related species (Dodd, 1957; Wanninger et al., 1999, 2000; Kay and Emlet, 2002). Further studies are required in order to establish if these results can be imputed to the necessity of improving the methodology or to biological constraints of the species.

Either way, the results of the present study could be considered as a first step towards the elaboration of a protocol that will permit to rear spats in laboratory conditions which could be used for further reintroduction and conservational projects.

ACKNOWLEDGMENTS

This work was funded by a postdoctoral grant of the Ministry of Education of Spain awarded to F. Espinosa (EX2006-0534), predoctoral grant of the Ministry of Education of Spain to G.A. Rivera-Ingraham (AP-2006-04220) and financial support by the "Autoridad Portuaria de Ceuta". The authors express their gratitude to "Consejería de Medio Ambiente-Obimasa," Ceuta, for its support.

LITERATURE CITED

Baker, M.C. and P.A. Tyler. 2001. Fertilization success in the commereial gastropod *Haliotis tuberculata*. Marine Ecology Progress Series 211: 205–213.

Dodd, J.M. 1957. Artifieial fertilisation, larval development and metamorphosis in *Patella vulgate* L. and *Patella caerulea* L. Pubblicazione di la Stazione Zoologiea di Napoli 29: 172–186.

Espinosa, F., J.M. Guerra-García, D. Fa, and J.C. García-Gómez. 2006. Aspeets of reproduetion and their implications for the conservation of the endangered limpet, *Patella ferruginea*. Invertebrate, Reproduction and Development 49: 85–92.

Frenkiel, L. 1975. Contribution à l'étude des cycles de reproduction des Patellidae en Algérie. Pubblieazione di la Stazione Zoologiea di Napoli 39: 153–189.

Guallart, J., J. Templado, M. Calvo, P. Cabezas, I. Acevedo, A. Maehordom, and A.A. Luque. 2006. Inventario y seguimiento de *Patella ferruginea* en España, así como la elaboraeión de una propuesta de estrategia de eonservación de la espeeie. Informe final. Ministerio de Medio Ambiente, Madrid, 199 pp.

Guerra-García, J.M., J. Corzo J., F. Espinosa, and J.C. García-Gómez. 2004. Assessing habitat use of the endangered marine mollusc *Patella ferruginea* (Gastropoda, Patellidae) in northern Africa: preliminary results and implieations for eonservation. Biological Conservation 116: 319–326.

IUCN (International Union for Conservation of Nature and Natural Resources). 1987. The IUCN position statement on traslocation of living organisms: introduetions, re-introductions and re-stocking. IUCN Coumeil, Gland, Switzerland, 4 September 1987, 20 pp.

Kay, M.C. and R.B. Emlet. 2002. Laboratory spawning, larval development, and metamorphosis of the limpets *Lottia digitalis* and *Lottia asmi* (Patellogastropoda, Lottiidae). Invertebrate Biology 121: 11–24.

Laborel-Deguen, F. and J. Laborel. 1991. Statut de *Patella ferruginea* Gmelin en Méditerranée. In: Boudouresque, C.F., M. Avon and V. Gravez (Eds.). Les Espèces marines à protéger en Méditerranée. GIS Posidonie Publishers, Marseille, pp. 91–103.

Ramos, M.A. 1998. Implementing the Habitats Directive for molluse speeies in Spain. Journal of Conchology Special Publieation 2: 125–132.

Templado, J. 2001. *Patella ferruginea* (Gmelin, 1791). *In*: Ramos, M.A., D. Bragado and J. Fernández (Eds.). Los invertebrados no inseetos de la Direetiva Hábitats en España. Ediciones Serie Técniea, Organismo Autónomo Parques Naeionales, Dirección General de Conservaeión de la Naturaleza, Ministerio de Medio Ambiente, Madrid, pp. 41–49.

Van Den Biggelaar, J.A.M. 1977. Development of dorsoventral polarity and mesentoblast determination in *Patella vulgata*. Journal of Morphology 154: 157–186.

Wanninger, A., B. Ruthensteiner, S. Lobenwein, W. Salvenmoser, W. Dietus, and G. Haszprunar. 1999. Development of the musculature in the limpet *Patella* (Mollusca, Patellogastropoda). Development, Genes and Evolution 209: 226–238.

Wanninger, A., B. Ruthensteiner, and G. Haszprunar. 2000. Torsion in *Patella caerulea* (Mollusea, Patellogastropoda): ontogenetic process, timing, and mechanisms. Invertebrate Biology 119: 177–187.